AD_____

Award Number: W81XWH-07-1-0062

TITLE: Cell fusion as a cofactor in prostate cancer metastasis

PRINCIPAL INVESTIGATOR: Yuri Lazebnik, Ph.D.

CONTRACTING ORGANIZATION:

Cold Spring Harbor Laboratory
Cold Spring Harbor, NY 11724

REPORT DATE: January 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

□

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
Jan 1, 2010	Annual	*4 DEC 2008 - 3 DEC 2009*

4. TITLE AND SUBTITLE
Cell fusion as a cofactor in prostate cancer metastasis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0062

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Yuri Lazebnik

Email: lazebnik@cshl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cold Spring Harbor Laboratory
Cold Spring Harbor, NY

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research and Materiel Command

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The research funded by this grant was proposed to test a hypothesis that cell fusion between tumor cells, or between tumor and normal cells contributes to metastasis. This contribution can be implemented by two mechanisms, by generating cells with diverse genetic and epigenetic properties, and by providing tumor cells with qualities of normal cells that are required to reside in normal tissues. This hypothesis might explain why cells tumor cells can grow at distant sites, why they express proteins that are normally expressed by cells of the metastasized tissue, and why only a minute fraction of cells released by the primary tumors form metastases. The specific aims were: To determine the mechanism of gene transfer between prostate cancer cells (Aim 1); and to determine whether cell fusion affects metastatic properties of prostate cancer cells (Aim 2). We hypothesized that gene transfer was mediated by cell fusion, but found that instead it was carried out by a retrovirus that the cells in question apparently acquired during propagation in mice. To accomplish Aim 2, we optimized production of cell hybrids by developing a new method for cell fusion and by dissecting how cell fusion induces pathways that cause apoptosis and prevent clonogenic cell survival. We also began conducted preliminary experiments to determine how cell fusion affects tumorigenicity. These experiments will continue during the extension that we were granted.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE		10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

INTRODUCTION: The main goal of the research funded by this grant was to test the hypothesis that fusion among tumor cells or fusion of tumor to normal cells, facilitates metastasis. The grant application was prompted by an observation (Glinsky et al., 2006) that injecting a mixture of human prostate cancer cells PC-3 that expressed either green or red fluorescent protein produced tumors composed of cells that expressed both proteins ("yellow" cells). The "yellow" cells had enhanced metastatic potential, which suggested that the horizontal exchange of the genetic information affected cell malignancy. We proposed to identify the mechanism of genetic exchange (Aim 1), with the main hypothesis being that this mechanism was cell fusion, and to test whether cell fusion can affect the ability of PC3 cells to metastasize (Aim 2).

KEY RESEARCH ACCOMPLISHMENTS:

AIM 1: "To determine the mechanism of gene transfer between prostate cancer cells". Our initial hypothesis was that the transfer of the genes encoding the fluorescent proteins was due to cell-to-cell fusion. However, our findings indicated a different and unexpected to us mechanism. We found that PC-3 released one or more infectious viruses that transferred the retroviral vectors that encoded RFP and EGFP in PC-3 cells. Since these vectors were based on mouse leukemia virus (MLV), we considered two closely related possibilities. One, that the virus released by PC-3 cells was a xenotropic mouse virus contracted during propagation of these cells in mice. Another, closely related hypothesis, was that experimental manipulations induced a human virus that is closely related to MLV, xenotropic murine leukemia virus (XMRV), which was recently identified in human prostate cancers (Urisman et al., 2006).

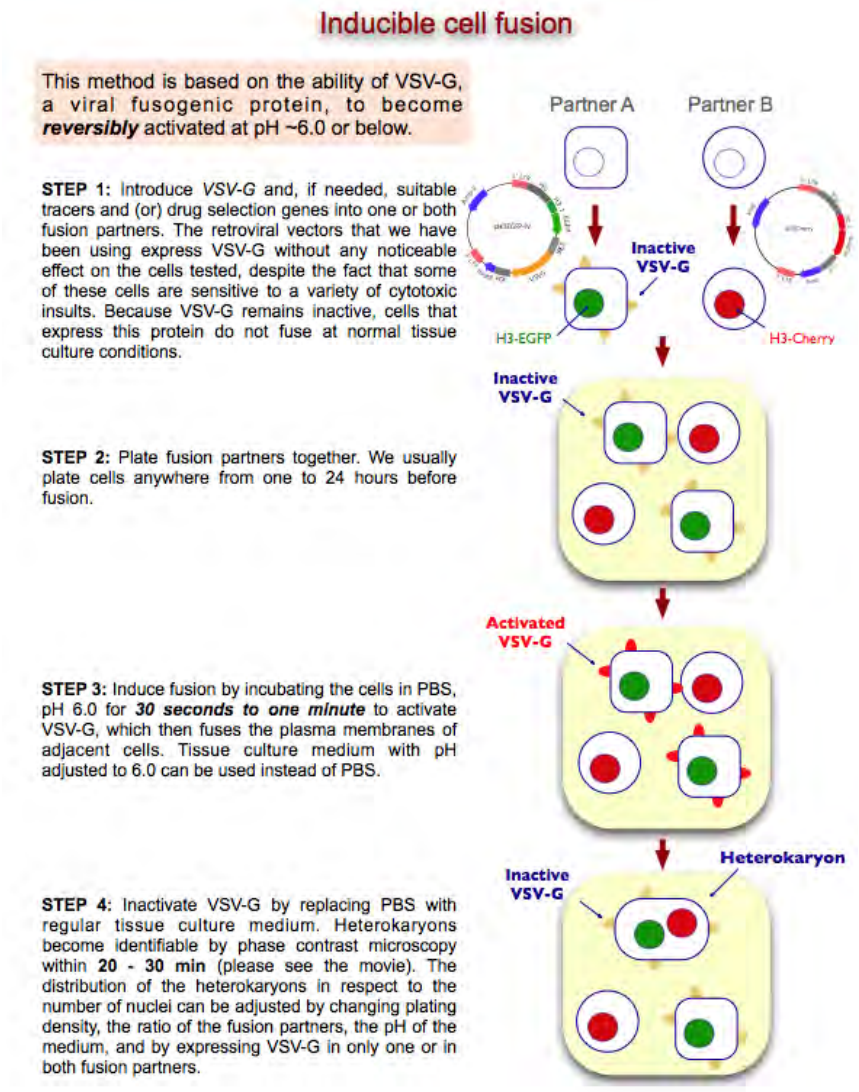

Figure 1. A method to induce cell fusion

To identify the virus, we isolated it from tissue culture medium conditioned by PC-3 cells that expressed by sequential centrifugation and analyzed the obtained samples by mass spectrometry. The resulting peptide sequences belonged to Gag and Env of the mouse leukemia virus (MuLV),

which was consistent with either of our hypotheses. We then obtained the viral DNA by RT-PCR and determined about 70% of its sequence. We found that the obtained sequence was not fully identical to any sequence in the non-redundant NCBI nucleotide database, but 97% to 98% identical to various MuLV or XRMV isolates. All detected mismatches were single nucleotide substitutions. Considering how highly similar the sequences of XMRV and MLV are, we could not identify the virus secreted by PC-3 cells unambiguously.

While were we conducting our studies, several reports questioned the link between XMRV and disease, including prostate cancer (Denner 2010). Furthermore, the failure to detect XMRV in European populations (Hohn et al., 2009) raised the possibility of artifacts that could explain finding this virus in human samples. Altogether, these reports, the findings that human xenografts can acquire mouse viruses (Voisset et al., 2008) and the need to focus our limited resources on the main problem – the potential role of cell fusion in cancer – led us to put further identification and characterization of the PC-3 virus on hold.

Overall, accomplishing Aim 1 had a major impact on our thinking and, consequently, affected our research. First, it brought to our attention the notion that viral infections can have unexpected consequences, including those that are related to carcinogenesis and tumor progression. Second, the opportunity to familiarize ourselves with the knowledge about MLV and XMRV prompted us to consider more systematically endogenous retroviruses as the primary suspects for agents that cause promiscuous cell fusion in the body. Finally, we also realized that introducing human cancer cells into mice could produce replicating viruses that can carry potentially dangerous genes and infect human cells.

AIM 2: To determine whether cell fusion affects metastatic properties of prostate cancer cells. The main goal of this aim was to test whether fusion of prostate cancer cells among themselves or to normal cells of the host affects the extent or tropism of metastasis. As we quickly found, accomplishing this Aim required first solving several technical and conceptual problems that we initially did not fully appreciate. Solving these problems provided us with better understanding of the consequences and outcomes of promiscuous cell fusion.

A new cell fusion approach. The finding (Aim 1) that retroviruses could transfer transgenes, implied that we could no longer use MPMV, a retrovirus that we used

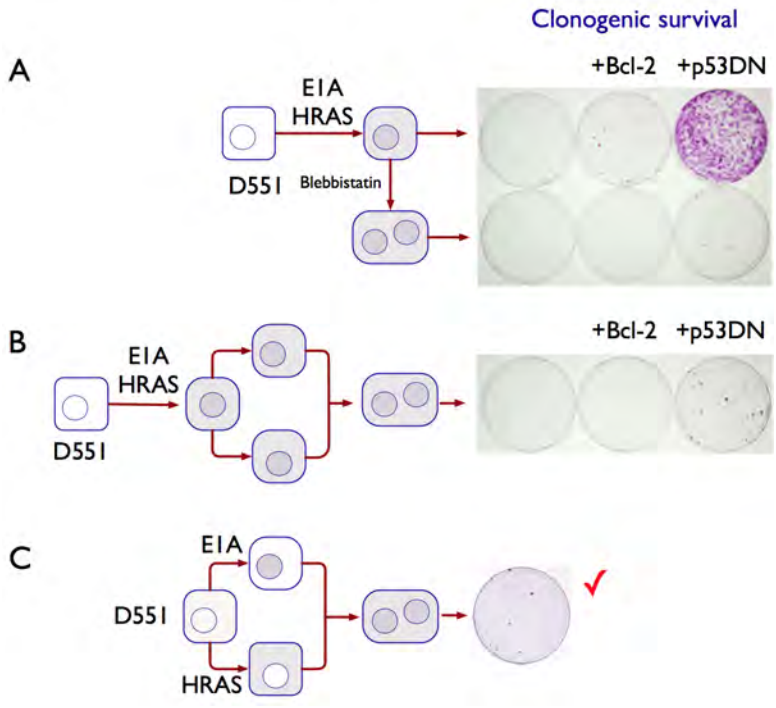

Figure 2. Distinct consequences of cell fusion and cytokinesis failure for clonogenic survival. D551 – normal diploid human fibroblasts Detroit 551.

routinely to fuse cells (Duelli et al., 2005; Duelli et al., 2007). The alternative approaches included the use of polyethylene glycol (PEG), which is notoriously toxic, or inactivated Sendai virus, which is laborious to obtain and expensive to buy. Therefore, we developed a new approach to fuse cells (Figure 1).

This approach takes advantage on the ability of VSVG, the fusogenic protein of the vesicular stomatitis virus (VSV), to be reversibly activated by a brief incubation in mildly acidic medium. The advantages of this method are that it is not toxic, even if used on cells that are highly sensitive to cytotoxic agents, it is easy to scale and automate, which makes it amenable to genetic and chemical screens, and it is convenient, inexpensive and reproducible. We anticipate that this method, once it is reported, will be used widely in research that either studies cell fusion, or uses this process as a tool to dissect other biological phenomena.

What are mechanisms that control the viability and clonogenic survival of cell hybrids? Our initial experiments to obtain clonogenic hybrids of PC-3 cells led us to realize that the mechanisms that control the fate of fused cells, and the fate of tetraploid cells in general, are poorly understood. To learn these mechanisms better, we turned to an experimental system that we used previously to study cell viability and to understand how or whether cell fusion can contribute to carcinogenesis (Duelli et al., 2005; Duelli et al., 2007). In this experimental system, normal diploid human fibroblasts are co-transduced with the adenoviral oncogene *E1A*, which deregulates cell cycle, and an oncogenic mutant of *RAS,* which has multiple effects, including inhibiting apoptosis caused by cell cycle deregulation. The resulting cells have epithelioid morphology, express markers of epithelial cells, proliferate in soft agar and, if provided with an additional oncogene, make tumors in nude mice. The use of normal diploid fibroblasts as the initial material and the use of defined oncogenes provided the benefit of using relatively well defined and homogeneous population of cells, which facilitated interpretation of the results.

To determine the fate of fused cells and to learn whether their fate is different from that of cells produced by cytokinesis failure, we compared effects of cell fusion and cytokinesis failure on cell viability and clonogenic survival. We found that either process caused apoptosis in a majority of the resulting binuclear cells, and delineated basic mechanisms through which apoptosis was induced. Remarkably, we found that tetraploid cells produced by either mechanism failed to produce clonogenic progeny (Figure 2). Preventing apoptosis by expressing Bcl-2 did not increase clonogenic survival of tetraploid cells, while expressing a dominant negative mutant of the tumor suppressor p53 did (Figure 2). These findings implied that tetraploidy induces more than one process that prevents clonogenic proliferation of these cells, and presented us with a paradox, as we were able to produce clonogenic hybrids using this experimental system previously (Duelli et al., 2005; Duelli et al., 2007). This paradox needed to be resolved as it promised either to provide new insights into regulation of clonogenic survival of tetraploid cells, or to reveal that we understood our experimental system not as well as we thought.

Some differences between the old and the new protocols suggested two explanations. One was that clonogenic survival depended on MPMV, the virus that we used previously to fuse cells. Another possibility was that previously, we transduced E1A and HRAS separately (Figure 2), and then fused the resulting cells,

while in the current experiments we co-transduced the oncogenes and then fused the resulting cells. To test these possibilities, we transduced E1A and HRAS separately (Figure 2C) and fused the resulting cells by the technique that we developed (Figure 1). We found that the resulting hybrids were clonogenic (Figure 2C), which indicated that cell fusion and cytokinesis failure have different potential to produce clonogenic tetraploid cells.

This conclusion might be relevant to an apparent contradiction raised by the tetraploidy model of carcinogenesis (Ganem et al., 2007). According to this model, conversion to tetraploidy is an intermediate stage in malignant transformation of solid tumors, even though a series of reports (Ganem et al., 2007), which are consistent with out own studies (Figure 2A,B) suggest that conversion to tetraploidy causes apoptosis and prevents clonogenic survival. Our findings suggest that how cells become tetraploid might determine whether they are capable of continuous proliferation. The experimental system that we have developed might help to understand better how the link between tetraploidy and cell cycle is regulated.

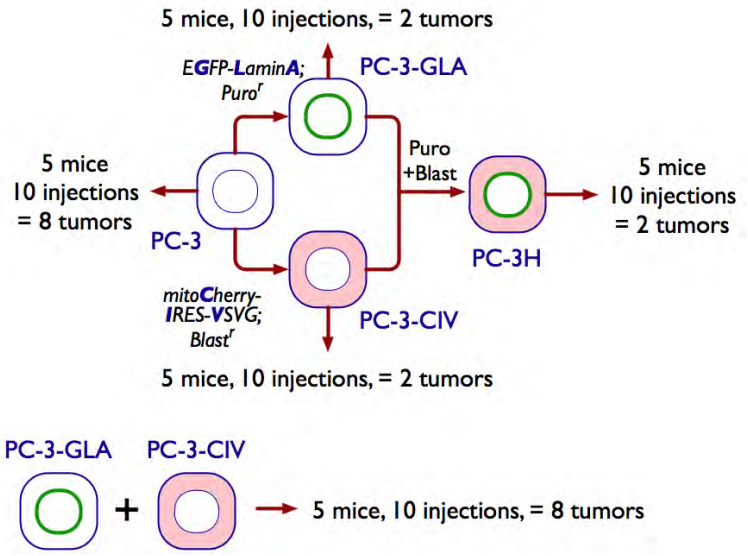

Figure 3. Comparing tumorigenicity of hybrids and the parental cell lines.

How does cell fusion affect malignant properties of cells? To begin addressing this question, which is central for the funded research, we obtained clonogenic hybrids of PC-3 cells (Figure 3). We injected these hybrids, the parental cell lines (PC-3-GLA and PC-3-CIV) that were made to produce and select the hybrids, and a mixture of PC-3-GLA and PC-3-CIV into the nude mice. We found that the hybrids produced as many tumors as the parental cell lines, but, to our surprise, the parental cells produced fewer tumors than the original PC-3 cells. This finding implied that introducing fluorescent tracers and drug selection genes somehow decreased tumorigenicity of the cells. We are currently devising a strategy to produce cell hybrids that would minimize the use of selection markers. Once this goal is completed, we will repeat and expand the experiment (Figure 3) and will analyze the resulting tumors in detail.

How does fusion to host cells affect malignant properties of cancer cells?

One hypothesis that underlied the proposed research was that fusion to host cells that move freely in the body, such as macrophages, can enable metastasis of tumor cells [Ref Duelli 2003]. To test this hypothesis, we developed an approach to fuse PC-3 to mouse macrophage progenitors (Figure 4), which were obtained through a collaboration with Dr. James Bliska (Stony Brook University). The resulting hybrids proliferate well, which proved that such hybrids can be generated. However, we delayed testing of their tumorigenicity as we

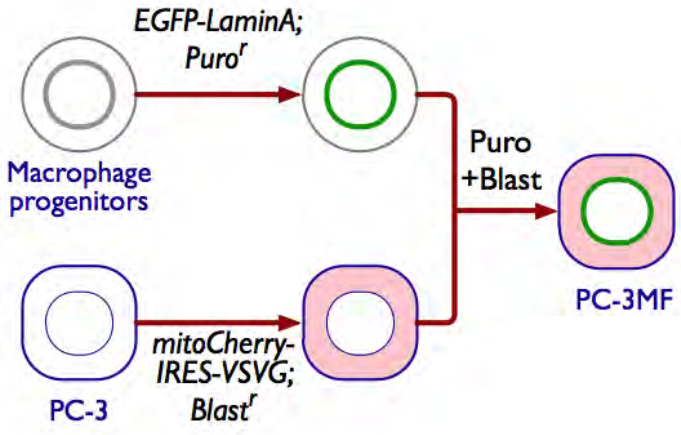

Figure 4. Generation of hybrids between PC-3 and macrophage progenitors

found that the fluorescence and drug selection markers that wer used decreased tumorigenicity of PC-3 cells. Once the alternative protocol for hybrid production is developed, we will resume this study.

REPORTABLE OUTCOMES: We developed an experimental system to identify pathways that control clonogenic expansion of tetraploid premalignant cells and generated a series of plasmids and cell lines that will be made available to the scientific community once our results are reported.

We also provided the scientific community with the review of evidence that underlies the proposed research (Duelli and Lazebnik, 2007). The unexpected finding that we encountered, especially the role of viruses in carcinogenesis, forced us to look more critically on the general concepts that underlie the current cancer research and on the concept of the hallmarks of cancer in particular. We came to the conclusion (Lazebnik 2010) that this concept needs to be reconsidered, which we will hope will help to focus cancer research more on understanding metastasis and tissue invasion, the true hallmarks of cancer.

CONCLUSIONS: Overall, by accomplishing a part of the proposed research, we unexpectedly entered an area of cancer biology – a relationship between viral infections and cancer. We will continue this research within the extension that we were granted and beyond.

REFERENCES:

Denner, J. 2010. Detection of a gammaretrovirus, XMRV, in the human population: open questions and implications for xenotransplantation. *Retrovirology*. 7:16.

Duelli, D., and Y. Lazebnik. 2007. Cell-to-cell fusion as a link between viruses and cancer. *Nat Rev Cancer*. 7:968-76.

Duelli, D.M., S. Hearn, M.P. Myers, and Y. Lazebnik. 2005. A primate virus generates transformed human cells by fusion. *J Cell Biol*. 171:493-503.

Duelli, D.M., H.M. Padilla-Nash, D. Berman, K.M. Murphy, T. Ried, and Y. Lazebnik. 2007. A virus causes cancer by inducing massive chromosomal instability through cell fusion. *Curr Biol*. 17:431-7.

Ganem, N.J., Z. Storchova, and D. Pellman. 2007. Tetraploidy, aneuploidy and cancer. *Curr Opin Genet Dev*. 17:157-62.

Glinsky, G.V., A.B. Glinskii, O. Berezovskaya, B.A. Smith, P. Jiang, X.M. Li, M. Yang, and R.M. Hoffman. 2006. Dual-color-coded imaging of viable circulating prostate carcinoma cells reveals genetic exchange between tumor cells in vivo, contributing to highly metastatic phenotypes. *Cell Cycle*. 5:191-7.

Hohn, O., H. Krause, P. Barbarotto, L. Niederstadt, N. Beimforde, J. Denner, K. Miller, R. Kurth, and N. Bannert. 2009. Lack of evidence for xenotropic murine leukemia virus-related virus(XMRV) in German prostate cancer patients. *Retrovirology*. 6:92.

Lazebnik, Y. 2010. What are the hallmarks of cancer? *Nat Rev Cancer*. 10:232-3.

Urisman, A., R.J. Molinaro, N. Fischer, S.J. Plummer, G. Casey, E.A. Klein, K. Malathi, C. Magi-Galluzzi, R.R. Tubbs, D. Ganem, R.H. Silverman, and J.L. DeRisi. 2006. Identification of a novel Gammaretrovirus in prostate tumors of patients
homozygous for R462Q RNASEL variant. *PLoS Pathog.* 2:0211-0225.

Voisset, C., R.A. Weiss, and D.J. Griffiths. 2008. Human RNA "rumor" viruses: the search for novel human retroviruses in chronic disease. *Microbiol Mol Biol Rev*. 72:157-96, table of contents.